Pulmonary immunization

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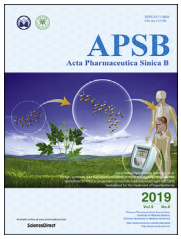

ORIGINAL ARTICLE

Pulmonary immunization: deposition site is of minor relevance for influenza vaccination but deep lung deposition is crucial for hepatitis B vaccination

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Abstract Vaccination *via* the pulmonary route could be an attractive alternative to parenteral administration. Research towards the best site of antigen deposition within the lungs to induce optimal immune responses has conflicting results which might be dependent on the type of vaccine and/or its physical state. Therefore, in this study, we explored whether deep lung deposition is crucial for two different vaccines, *i.e.*, influenza and hepatitis B vaccine. In view of this, influenza subunit vaccine and hepatitis B surface antigen were labeled with a fluorescent dye and then spray-dried. Imaging data showed that after pulmonary administration to mice the powders were deposited in the trachea/central airways when a commercially available insufflator was used while deep lung deposition was achieved when an in-house built aerosol generator was used. Immunogenicity studies revealed that comparable immune responses were induced upon trachea/central airways or deep lung targeting of dry influenza vaccine formulations. However, for hepatitis B vaccine, no immune responses were induced by trachea/central airways deposition whereas they were considerable after deep lung deposition. Thus, we conclude that deep lung targeting is not a critical parameter for the efficacy of pulmonary administered influenza vaccine whereas for hepatitis B vaccine it is.

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1. Introduction

The parenteral route is considered to be the gold standard for vaccination[1−4]. However, there are certain drawbacks associated with parenteral immunization. Challenges such as needle phobia, pain and redness at the site of injection and transmission of infectious diseases due to needle stick injuries are some of the issues related to parenteral immunization. Besides, for diseases such as influenza, that do spread *via* the respiratory tract, parenteral immunization fails to elicit a potent local mucosal immune response[5,6]. Hence, for such infectious diseases, respiratory tract administration might be an attractive alternative to parenteral administration. Even for non-airborne transmitted infectious diseases, for example hepatitis B, respiratory tract immunization, particularly *via* the pulmonary route, might be advantageous due to its non-invasive nature, large surface area for immune response induction and presence of abundant antigen presenting cells.

It can be hypothesized that the efficacy of immunization *via* the pulmonary route depends on number of factors including the site of deposition within the respiratory tract as well as the type of vaccine used. For diseases that do spread *via* the respiratory tract such as influenza and tuberculosis, both upper/central airways and deep lung deposition led to the development of considerable immune responses. However, Minne et al.[7] and Todoroff et al.[8] claimed with a liquid formulation that deep lung deposition was superior in terms of induction of immune responses. Despite that Bhide et al.[9] recently demonstrated in cotton rats that the site of deposition of influenza vaccine within the respiratory tract holds minor relevance in the protective efficacy against a challenge with a live virus. However, in the study of Bhide et al.[9] deep lung deposition was achieved with a liquid formulation, while trachea/central airways deposition was achieved with a powder formulation[9]. Thus, the physical state of the vaccine might also have played a role in this study. Therefore, it is vital to further investigate whether the same conclusion holds true when powders are targeted deep into the lungs. Furthermore, it is unknown whether the site of deposition within the respiratory tract is of importance for diseases that do not spread *via* the respiratory tract such as hepatitis B. In principle, however, the antigen should be targeted to that part of the respiratory tract where there is an optimal induction of immune responses. The site of antigen deposition within the respiratory tract will be influenced by the physical form of the formulation, the delivery device used for administration and for human vaccination also the inhalation manoeuvre. Vaccines for pulmonary administration can be formulated as liquids and as dry powders[10−12]. However, due to their long term stability at ambient temperature, ease in stockpiling and possibility of being readily available for mass vaccination, dry powder vaccine formulations are preferred[13,14]. For deep lung deposition, a dry powder formulation with an aerodynamic particle size distribution of 1−5 μm is required. In addition, a delivery device is required that does not only efficiently disperse the powder particles in the inhaled air stream but also delivers the aerosol particles at a low velocity. High velocity and large size particles would either be exhaled or deposited in the upper airways. For animal studies, the only commercially available (now discontinued) delivery device is an insufflator developed by Penn-Century (Penn-Century, Wyndmoor, USA). Unfortunately, this device has shown to deliver large agglomerates of powder particles at a high velocity, thereby depositing powder particles only in the trachea/central airways and not in the deep lungs[9,15,16]. A newly developed device, PreciseInhale, has shown to cause less tracheal deposition as compared to the insufflator[17]. However, one of the major limitations associated with this device is the use of high-pressure pulse for the dispersion of dry powders. In a study of Lexmond et al.[18], this resulted in the crystallization of amorphous spray-dried products thereby forming large agglomerates. As a consequence, the emitted fraction was extremely low (<1%). Recently, Tonnis et al.[16] described an in-house built aerosol generator for pulmonary administration of dry powders to animals. Approximately 17% of the loaded spray-dried product was emitted from the loading chamber, hence it is assumed that the aerosol generator did not affect the amorphous state of the powder during dispersion. In addition, the aerosol generator was found to be suitable to target lower regions of the respiratory tract, *i.e.*, deep lungs. Thus, by using the insufflator and the aerosol generator, targeting dry powder formulations to the trachea/central airways and to the deep lungs, respectively, is feasible. This enables research regarding the preferred site of antigen deposition within the respiratory tract for different vaccines.

The aim of the current study was to investigate the effect of the site of antigen deposition within the respiratory tract on the immune response in mice. For this, influenza and hepatitis B vaccine liquid formulations were fluorescently labeled, spray-dried into powder formulations and targeted to different regions of the respiratory tract of mice. Imaging system was used to verify trachea/central airways deposition by insufflator and deep lungs deposition by the aerosol generator. Immune responses induced by the spray-dried formulations deposited in different parts of the respiratory tract were compared to investigate the best site that should be targeted for a particular vaccine candidate.

2. Materials and methods

2.1. Vaccines

Influenza subunit vaccine (A/California/7/2009 H1N1) was kindly provided by Abbott, Weesp, Netherlands and hepatitis B surface antigen was a generous gift from Serum Institute of India, Pune, Maharashtra, India.

2.2. Labeling of influenza and hepatitis B vaccines

Influenza and hepatitis B vaccines were fluorescently labeled with a near-infrared fluorescent dye VivoTag 680XL (PerkinElmer, Walthman, MA, USA) *as per* the instructions provided by the manufacturer. Briefly, 1 mL of influenza subunit vaccine (153 μg/mL) was mixed with 4.6 μL of Vivo Tag (25 mg/mL) and 100 μL of 1 mol/L $NaHCO_3$ solution. For hepatitis B, 91 μL of the vaccine solution (1.1 mg/mL) was mixed with 1.5 μL of Vivo-Tag (25 mg/mL) and 10 μL of 1 mol/L $NaHCO_3$ solution. After 2 h of constant shaking at room temperature, the unbound fluorophore

was removed by passing the mixtures through Zeba Spin Desalting Columns (ThermoScientific, Rockford, IL, USA). In order to calculate the degree of labeling, the concentration of labeled vaccine and dye was determined at 280 and 668 nm, respectively. Fluorophore cross-talk at 280 nm was adjusted by subtracting 16% of the absorbance at 668 nm from the absorbance at 280 nm. It was calculated that on average 4 or 3 dye molecules were bound to each influenza and hepatitis B vaccine molecule, respectively.

2.3. Spray drying

Labeled and unlabeled liquid influenza and hepatitis B vaccine formulations were spray-dried together with inulin (4 kDa, Sensus, Roosendaal, Netherlands), which was used as a stabilizer/bulking agent. A Büchi B-290 Mini Spray Dryer (Büchi, Flawil, Switzerland) equipped with a standard two fluid nozzle was used to spray dry liquid vaccine formulations. The mixture of liquid vaccine formulations and inulin (prepared in distilled water) was formulated (influenza subunit vaccine:inulin 1:100 (*w*/*w*); hepatitis B surface antigen:inulin 1:250 (*w*/*w*)) to obtain a final solid concentration of 50 mg/mL. The mixture was pumped at a speed of 5 mL/min and at an inlet temperature of 100 °C leading to an outlet temperature of 58 °C. An atomizing airflow was set to 500 L_n/h with an aspirator setting of 100%.

2.4. Scanning electron microscopy

Scanning electron microscopy was done as described previously[19]. Briefly, images were captured using a JEOL JSM 6301-Microscope (JEOL, Tokyo, Japan). Vaccine powders were placed on a carbon tape on a metal disc and coated with 30 nm of gold/palladium using a Leica EM LCD250 sputtering device (Leica Microsystems, Rijkswijk, the Netherlands).

2.5. Laser diffraction

The primary particle size distribution of spray-dried powders was determined by laser diffraction analysis. A pressure of 1 bar was used to disperse spray-dried powders using a RODOS disperser (Sympatec, Clausthal-Zellerfeld, Germany). The start of the measurements was triggered on an optical signal of 0.2% on channel 30, and the measurements were stopped either after the signal decreased to a value lower than 0.2% on the same channel for a period of 1 s, or after 3 s of real measurement time. A 100 mm lens was used and the particle size distribution was calculated using the Fraunhofer theory.

The particle size distribution of the powders was also analyzed after dispersion of powders by the insufflator (model DP-4M; Penn-Century, Wyndmoor, PA, USA) and aerosol generator. The particle size dispersed from the insufflator was determined according to the procedure of Hoppentocht et al.[20] Briefly, the insufflator was mounted on an in-house mounting plate and loaded with 1 mg of spray-dried powder. The tip of the insufflator was placed in the front of laser beam and the powders were dispersed using an air pulse of 200 μL using an AP-1 air pump (Penn-Century). Like dispersion from the RODOS, a similar 100 mm lens was used.

The particle size distribution of the powders dispersed from the aerosol generator was determined by dispensing powders in the collection chamber of the aerosol chamber. A fraction of these powders was allowed to escape through a small tube with its tip placed in the front of laser beam (see following section "*in-vivo* deposition study").

2.6. In vivo deposition study

2.6.1. Powder administration by insufflator and the aerosol generator

Animal experiments were approved by The Institutional Animal Care and Use Committee of the University of Groningen, Groningen, The Netherlands (permit number: 6870AE). The animals were administered with labeled vaccine powders only once. For each vaccine candidate, 15 specific pathogen-free 8−10 week-old BALB/c mice (Envigo, Horst, the Netherlands) were randomly divided into three groups with 6 animals/group except for control, which had 3 animals/group. Mice were anaesthetized by an intraperitoneal injection of ketamine and dexdormitor and were intubated with an AutoGrade catheter (20G, BD Insyte™ Autoguard, BD, Breda, the Netherlands). For the administration of dry powders to trachea/central airways, the insufflator was loaded with 1 mg of labeled spray-dried powders containing either 10 μg influenza subunit vaccine or 4 μg of hepatitis B surface antigen. The delivery tube (internal diameter: 0.5 mm) was passed through the trachea and the tip of the delivery tube was placed at the bifurcation of trachea, *i.e.*, carina. An AP-1 air pump was used to disperse powders with three puffs of 200 μL of air/puff in approximately 2 s. Thus, dispersion in this way led to a flow rate of 0.1×10^{-3} L/s.

The amount of the powder that was dispersed from the insufflator was determined by weighing the insufflator before and after administration. It was found that 0.81 ± 0.27 mg (average ± standard deviation) of spray-dried powder was dispersed from the insufflator.

The pictorial representation of assembly and administration by aerosol generator has been shown by Tonnis et al.[16] Powders were administered using the similar procedure as described by Tonnis et al.[16] Briefly, this aerosol generator contains an air classifier as the powder disperser. The powder compartment of the aerosol generator was filled with approximately 180 mg of spray-dried vaccine formulation and was positioned on top of the air classifier, thereby closing the air inlet to the aerosol generator. Thereafter, the pressure inside the aerosol generator was lowered after which the valve to the vacuum pump was closed. Next, the air inlet was opened to allow air to flow freely through the powder compartment into the air classifier and finally into the particle collection chamber. Aerosols were generated and the intubated mouse was then connected to the aerosol generator *via* an intubation needle (internal diameter: 1.1 mm). A syringe was used to deliver 3 mL of air during the time period of 1 min; this air was introduced to deliver aerosols from the aerosol chamber to the lungs of intubated mouse. The delivery of 3 mL of air in 1 min corresponds to a flow rate of 0.5×10^{-4} L/s which is 2 times less than the flow rate determined from the insufflator (0.1×10^{-3} L/s). In addition, the internal diameter of the delivery tube used for insufflator (0.5 mm) was 2.2 times smaller than that used for aerosol generator (1.1 mm). This means that the aerosol generator tubing has 4.8 times larger cross-sectional area for powder flow than the tube of the insufflator. Hence, the cumulative effect of 2 times slower flow rate of aerosol generator and 4 times greater cross-sectional flow area implies that the particles dispersed from the aerosol generator travelled with 9.7 times lower velocity than those dispersed from the insufflator.

For the determination of dose dispersed from the aerosol generator, the powder residues that remained in the dose compartment, air classifier and aerosol chamber were collected. It was found that 0.56 ± 0.11 mg (average ± standard deviation) of the spray-dried powders were emitted from the aerosol generator.

2.7. *In vivo imaging system (IVIS)*

Immediately after administration of fluorescently labeled vaccine by the insufflator or aerosol generator, animals were sacrificed and their lungs along with trachea were taken out. Lungs were either used as such or dissected into lung lobes. The entire lungs or dissected lung lobes were placed in petri-dishes and the emitted fluorescent signal was determined by IVIS (PerkinElmer, Waltham, MA, USA). An excitation wavelength of 675 nm was used to measure fluorescent emission at 720 nm. The intensity of the emitted light (photons/s/cm^2/steradian) was quantified using Living Image Software v3.2 (PerkinElmer, Waltham, MA, USA). Fluorescent intensities determined in the lungs of non-vaccinated animals (negative control) were subtracted from the fluorescent intensities of vaccinated animals. Due to anatomical proximity of heart with lungs, hearts of vaccinated as well as non-vaccinated animals were also taken out to determine whether the administration was indeed successful in lungs.

To further quantify the deposition of powder vaccine formulations in trachea/central airways *vs.* deep lungs, each lung lobe was divided into two equal halves by area with a region of interest circular in shape. No clear distinction is described in the literature in between central airways and deep lungs of mice, therefore, this region of interest of specific dimensions (described as above) was used for all vaccinated as well as non-vaccinated animals. The sum of the fluorescent intensities emitted from the central parts of lung lobes along with trachea was taken as trachea/central airways. For deep lungs, the sum of the fluorescence intensity of a region of interest targeting central airways was subtracted from the total fluorescence intensity of all the lung lobes.

2.8. *Immunization and sample collection*

For each vaccine candidate, 21 specific pathogen-free 8−10 week-old BALB/c mice (Envigo) were randomly divided into 4 groups with 6 animals/group except for control which had 3 animals/group. Mice were anaesthetized by an intraperitoneal injection of ketamine and dexdormitor and were intubated with an AutoGrade cathether (20G, BD Insyte™ Autoguard, BD, Breda, The Netherlands). Animals were immunized twice, *i.e.*, on day 0 and day 14 with unlabeled vaccine powders.

After immunization, antisedan was administered subcutaneously for awakening of the animals. After awaking, mice were kept in a recovery incubator with a temperature of 25 °C for 2 h. The same procedure was repeated on day 14. On day 14 and day 28, blood was collected by cheek puncture and cardiac puncture, respectively. On day 28, lung washes were collected by flushing lungs with 1 mL of PBS containing complete protease inhibitor cocktail tablets (Roche, Almere, the Netherlands).

2.9. *ELISA*

For influenza, sera and lung washes were used for the determination of influenza specific IgG and IgA titers. ELISA plates (Grenier Bio-One, Alphen aan den Rijn, the Netherlands) were coated overnight with 500 ng/well of subunit influenza vaccine (A/california/H1N1) at 37 °C. ELISA was performed as described previously[21]. Synergy HT plate reader (BioTek, Winooski, VT, USA) was used to measure absorbance at 492 nm. Average IgG serum titers were calculated by determining the $\log_{10}$ of the reciprocal of the sample dilution corresponding to an absorbance of 0.2 at 492 nm. IgA and IgG levels in bronchioalveolar lavages (BAL) are presented as average of the absorbance at 492 nm for undiluted lung washes. For hepatitis B, sera were used for the determination of hepatitis B specific IgG, IgG1 and IgG2a antibody titers. ELISA plates were coated overnight at room temperature with 0.2 μg of hepatitis B surface antigen/*per* well. The ELISA procedure was followed as described by Hirschberg et al.[22] Absorbance was measured at 415 nm and titers were calculated as the reciprocal of the sample dilution corresponding to an absorbance of 0.2 at 415 nm. For BAL IgG, average absorbance measured at 415 nm for undiluted lung washes was plotted.

2.10. *Statistics*

Mann−Whitney U test (two tailed) was used to test if the differences between two groups tested for different parameters were significant. A P value of less than 0.05 was considered significant. P values less than 0.05, 0.01, and 0.001 are denoted by *, ** and ***, respectively. Graphs were plotted using GraphPad Prism 5 software (GraphPad Software, La Jolla, San Diego, CA, USA).

3. Results

3.1. *Physical characteristics of vaccine powders*

The primary particle size distribution and the morphology of influenza and hepatitis B powders were determined. Laser diffraction analysis following dispersion by RODOS showed that both powder formulations had median diameters (X_{50} values) between 2.2 and 2.7 μm (Table 1) and 90% total powder volume (X_{90}) was in particles smaller than 6 μm. The primary particle size distribution was compared to the particle size obtained by dispersion from the insufflator and the aerosol generator. When these powders were dispersed from the insufflator and aerosol generator, the average geometric particle size was found to be 4.7−5.5 μm and 7.3−9.1 μm, respectively (Table 1). X_{50} values of the powders dispersed from aerosol generator were found to be larger than that of insufflator. Apparently, agglomerates were broken up to a certain extent in both cases, but with insufflator more efficiently. However, X_{90} values of the powder particles dispersed from insufflator and aerosol generator were found to be comparable (20.5−21.2 μm for the aerosol generator and 20.7−21.2 μm for the insufflator) (Table 1). This implies that majority of the particles (X_{90}) had size $\leq$ 21.2 μm both for the insufflator and the aerosol generator.

Scanning electron microscope images show that the spray-dried vaccine powders had intact spherical particles (Fig. 1A and B).

3.2. *In vivo deposition study*

To assess the deposition of powder formulations dispersed from the insufflator and aerosol generator in the trachea/lung lobes of mice, imaging was performed on intact as well as dissected lung lobes. A schematic diagram of trachea and lung lobes highlighting central airways and deep lungs is shown in Fig. 2A. IVIS images

Table 1 Laser diffraction particle size distributions of labeled- and unlabeled-vaccine powders dispersed from RODOS, insufflator and aerosol generator.

Disperser	Geometric particle size					
	Influenza			Hep-B		
	X10	X50	X90	X10	X50	X90
RODOS	0.9±0.04	2.2±0.1	5.5±0.06	1.2±0.07	2.7±0.09	5.8±0.1
Insufflator	1.5±0.02	4.7±0.05	20.7±0.07	1.6±0.02	5.5±0.02	21±0.03
Aerosol generator	2.1±0.04	7.5±0.09	20.5±0.03	2.4±0.05	8.9±0.1	21.2±0.05

Data are average ± standard error of the mean ($n = 9$).

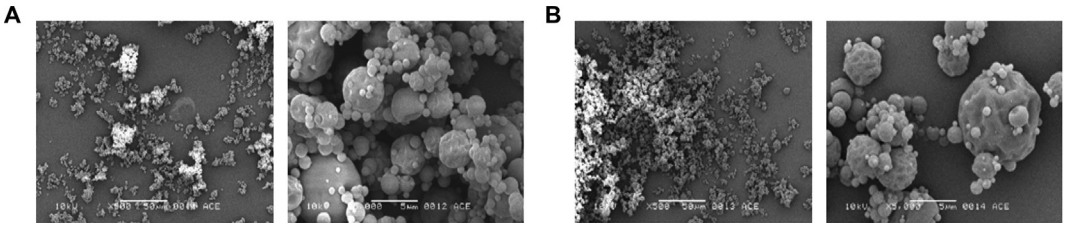

Figure 1 Physical characteristics of vaccine powders. Scanning electron microscope images of spray-dried influenza (A) and hepatitis B (B) vaccine powders at a magnification of 500 × (Scale bar = 50 μm) and 5000 × (Scale bar = 5 μm).

of the intact lungs show that the major site of powder deposition upon dispersion from the insufflator is the trachea as it showed bright yellow fluorescent spots (Fig. 2B and D). Upon dissection of lungs into trachea and individual lung lobes, a small amount of the powder also seemed to be deposited in the central parts of lung lobes (Fig. 2B and D). However, when the powders were dispersed from the aerosol generator, fluorescence signals of about the same intensity were emitted from the entire lungs (Fig. 2B and D). Further, upon dissection of the lungs, comparatively, a more homogenous distribution of powder could be determined in trachea and lung lobes; no bright yellow fluorescent spots were visible throughout the lung (Fig. 2B and D).

Upon quantification, it was found that both influenza and hepatitis B powder vaccine formulations, upon dispersion from the insufflator, were deposited in the trachea/central airways (100%). Not even a minor fraction of the powder particles did seem to penetrate into the peripheral parts of the lungs (Fig. 2C and E). However, when these powders were dispersed from the aerosol generator, significantly lower amounts of powders, approximately 55%−58%, were found to be deposited in the trachea/central airways whereas the amount that penetrated the deep lung was found to be about 42%−45% (Fig. 2C and E). Hence, with the aerosol generator, a homogenous distribution of powder formulations between the trachea/central and peripheral airways could be obtained (Fig. 2C and E). These findings are in line with the results obtained by Tonnis et al.[16] who administered labeled ovalbumin powders to mice using the insufflator and aerosol generator.

3.3. *Influence of site of deposition on immune responses: influenza*

The influence of deposition of influenza vaccine formulations in trachea/central airways and deep lungs was investigated by evaluating systemic and mucosal immune responses against influenza subunit vaccine. Lung washes were collected on day 28 whereas sera were taken both on day 14 and day 28. The intramuscular (i.m.) group was used as the positive control whereas untreated animals were the negative control group. On day 14, majority of the vaccinated animals had developed anti-influenza serum IgG titers. It was found that influenza vaccine powders targeted to trachea/central airways by the insufflator led to the development of comparable serum IgG titers as deep lung targeted vaccine administered *via* the aerosol generator (Fig. 3A). Likewise, upon evaluation of serum IgG titers at day 28, considerable IgG titers of similar magnitude were found for animals vaccinated with the insufflator and aerosol generator (Fig. 3B).

Lung washes collected on day 28 were evaluated for IgG as well as IgA titers. Immunization with powder formulations *via* the pulmonary route led to the development of low IgG titers; however, these were found to be equivalent for powder formulations administered *via* the insufflator or aerosol generator (Fig. 3C). In addition, lung IgA titers of comparable magnitude were found in mice treated with influenza subunit vaccine powders targeted to trachea/central airways or those treated with vaccine targeted to the deep lungs (Fig. 3D). As expected, no lung IgA titers could be detected by i.m. vaccination with influenza (Fig. 3D) which is in line with earlier reports[12,19,23].

3.4. *Influence of site of deposition on immune responses: hepatitis B*

The influence of hepatitis B powder deposition in the trachea/central airways *vs.* deep lung on the immune responses was evaluated. Systemic immune responses were evaluated both on day 14 and day 28. On day 14, only 2 out of 12 animals pulmonary immunized with powder formulation of hepatitis B, had developed serum IgG titers (Fig. 4A). For these 2 animals, powder vaccine was targeted deep into the lungs using the aerosol generator. None of the animals that had trachea/central airways deposition achieved by insufflator, had any IgG titers in their sera (Fig. 4A). Even on day 28, none of the animals vaccinated with insufflator developed serum IgG titers. On the contrary, all animals vaccinated with aerosol generator had

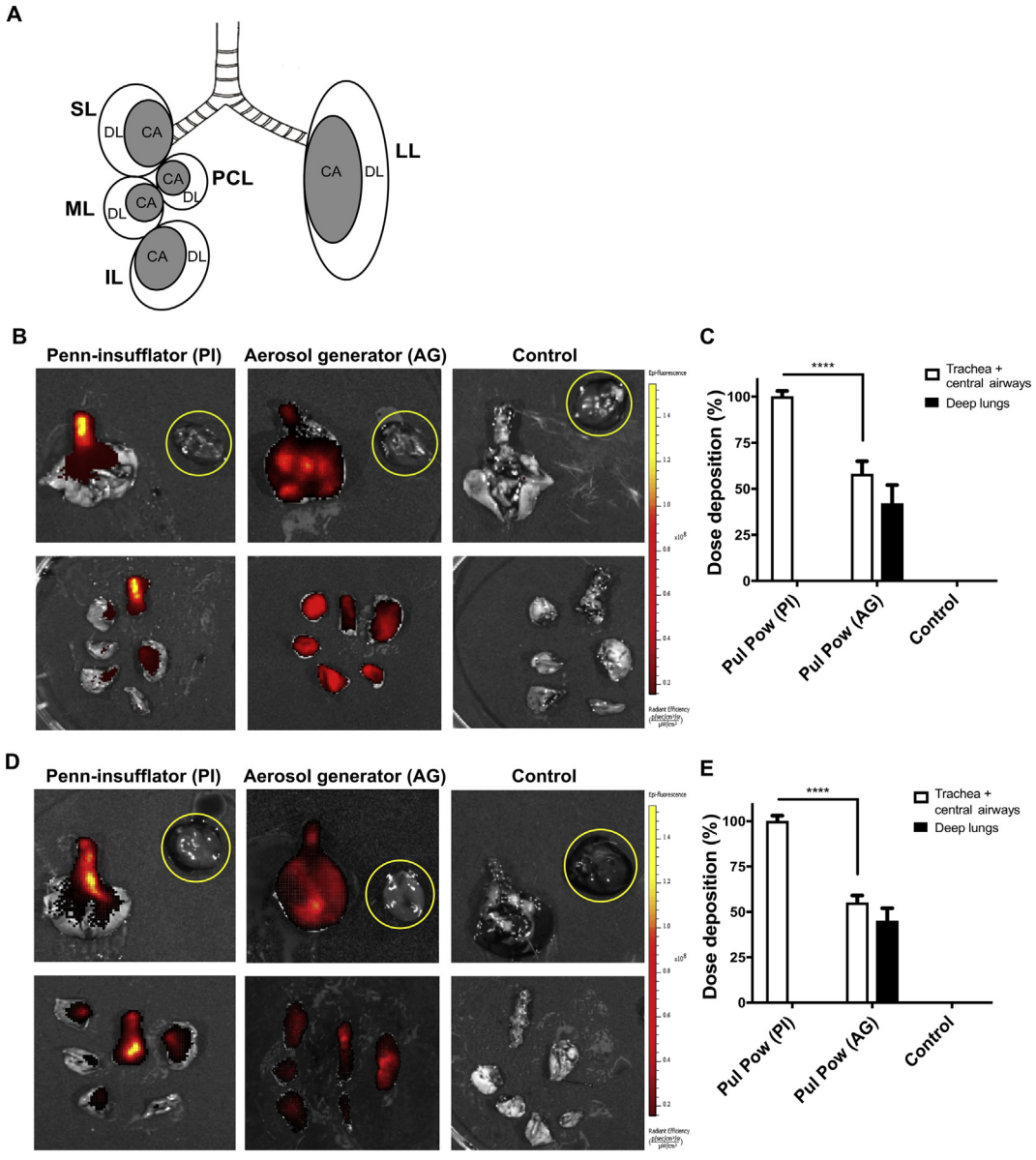

Figure 2 *In-vivo* deposition of dry powder vaccine formulations dispersed from the insufflator and the aerosol generator. Labeled vaccine powders were pulmonary administered (Pul Pow) to mice by the insufflator or aerosol generator. Immediately after administration, mice were sacrificed to image intact lungs as well as dissected lung lobes. (A) Schematic representation of trachea and all the lung lobes (LL: left lung lobe; SL: superiorlobe; ML: middle lobe; IL: inferior lobe; PCL: post caval lobe) showing central airways (CA) and deep lung (DL). Representative IVIS images of the intact as well as dissected lungs of animals that had received influenza (B) and hepatitis B (D) powders from insufflator or aerosol generator or were left untreated. Untreated animals were used as negative control for imaging calculations and heart was used as an anatomical negative control due to its proximity to lungs. Quantification of deposition of influenza (C) or hepatitis B (E) powder formulations in trachea/central airways versus deep lungs of animals ($n = 6$). Data are presented as average±standard error of the mean. Levels of significance are denoted as $^{****}P \leq 0.0001$.

developed considerable serum IgG titers after 28 days (Fig. 4B). Upon further quantification of serum IgG1 or IgG2a titers, a similar trend could be noticed (Fig. 4C). Pulmonary immunization with hepatitis B powders dispersed by the aerosol generator induced both IgG1 and IgG2a titers; as expected no such titers could be determined for the powders dispersed by the insufflator (Fig. 4C).

IgG titers were further determined in the lung washes of these animals. Lung IgG titers were found to be in line with serum IgG titers. No lung IgG titers were found in the lungs of animals vaccinated with insufflator whereas the animals vaccinated with aerosol generator had considerable lung IgG titers (Fig. 4D).

4. Discussion

In the current study, we investigated the influence of the deposition site of pulmonary administered powder vaccine candidates on the immunogenicity evoked by these formulations. Powder vaccine formulations of influenza and hepatitis B were targeted to different regions of the respiratory tract by using the insufflator

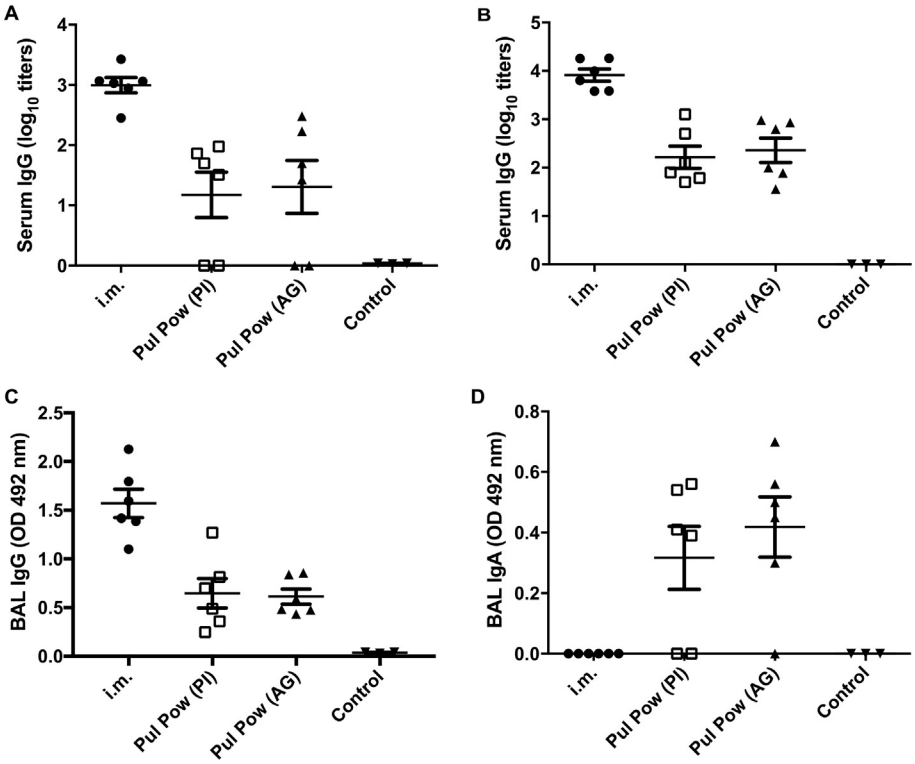

Figure 3 Immune responses induced by dry powder influenza vaccine formulations administered by the insufflator and the aerosol generator. Mice were immunized twice on day 0 and day 14 with 5 μg of influenza subunit vaccine powder formulation by intramuscular (i.m.) or pulmonary route. Pulmonary powder formulations were administered by Penn-Insufflator [Pul Pow (PI)] or aerosol generator [Pul Pow (AG)]. Immune responses were determined on day 14 and day 28. Serum IgG titers on day 14 (A) and on day 28 (B). (C) Lung IgG titers; (D) Lung IgA titers. Data are presented as average±standard error of the mean ($n=6$).

and an in-house built aerosol generator. The deposition of powder formulations in different regions of the respiratory tract was confirmed by an imaging system. The magnitude of immune responses evoked by these differently targeted/deposited powder formulations were compared to each other.

Powder administration using the insufflator resulted in deposition in the trachea/central airways whereas administration *via* the aerosol generator resulted in a homogeneous distribution throughout the entire lungs. Since the median diameter (X_{50}) of the particles dispersed from the aerosol generator was larger than those dispersed from the insufflator, in principle, the opposite would be expected in terms of deposition. However, the observed differences in powder deposition can be ascribed to the differences in the velocity by which the powder particles were dispersed from these two different devices. According to manufacturer's instructions, the insufflator's tip was placed at the bifurcation of trachea, *i.e.*, the carina. Hence, it can be expected that the powder particles would have been deposited throughout the whole lung. However, during insufflation, powder particles were deagglomerated and dispersed with 200 μL of air in a single step within about 2 s. Therefore, the air was introduced at a high velocity to an already inflated lung, which most likely resulted in a return flow of air containing powder particles. As a result, these particles were either deposited in the trachea or exhaled (visual observation).

By contrast, pulmonary administration by aerosol generator is a two-step process. In the first step, powder particles were deagglomerated by allowing the air to enter from the top of the compartment, which allowed the production of an aerosol in the collection chamber. In the second step, with the introduction of 3 mL of air, powder particles were delivered to the lungs slowly (1 min) and were being deposited in the lungs of the animals. In comparison with the insufflator, the velocity of the powder particles dispersed from the aerosol generator was almost 10 × slower (see experimental section). Thus, the return flow of air was minimized and as a consequence the amount of powder vaccine deposited in the trachea was found to be quite low. In addition, the much lower velocity of the powder particles dispersed by the aerosol generator most likely overcompensated the fact that the particle size distribution (X_{50}) was larger than that of the dry powder dispersed by the insufflator.

Immunogenicity studies in mice showed the induction of immune responses of comparable magnitude upon deposition of influenza vaccine powders either in trachea/central airways or in the deep lungs. In our previous studies, we found comparable immune responses induced by pulmonary administration of liquid and powder influenza vaccine formulations[11,12]. Our another study showed that liquid influenza vaccine formulation deposits deep in the lungs of cotton rats whereas the powder formulation is deposited in the trachea/central airways upon administration using the insufflator[9]. Although this study by Bhide et al.[9] demonstrated differences in deposition, comparable protective efficacy was induced by liquid and powder influenza vaccine formulations deposited deep in the lungs or trachea/central airways, respectively[9]. Consistent with the finding with liquid and powder in our previous study, we have found in this study that influenza vaccine

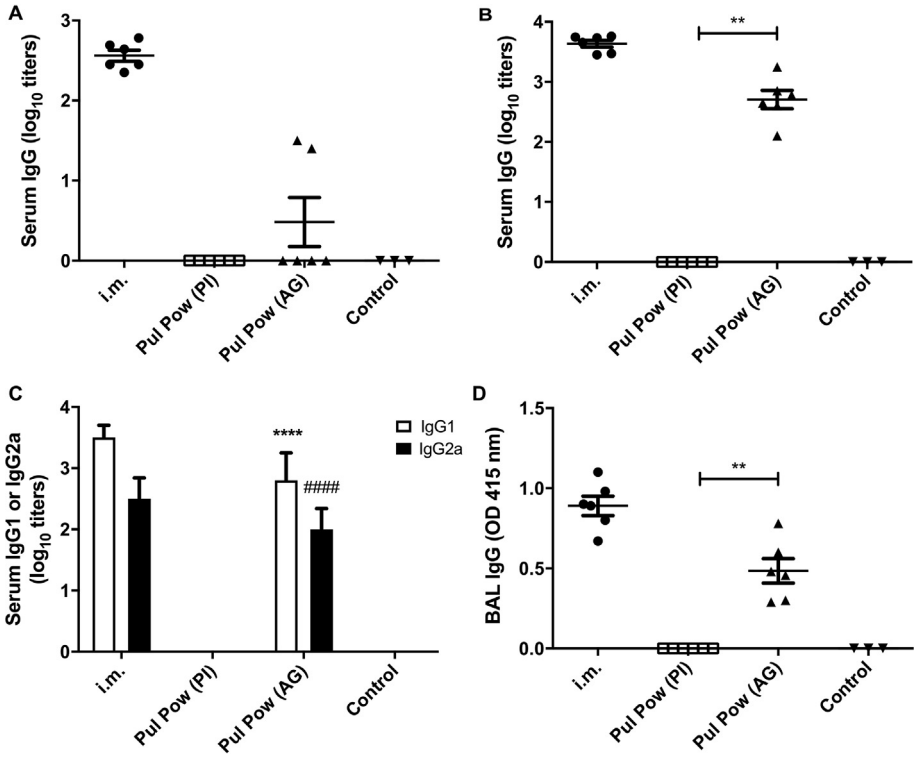

Figure 4 Immune responses induced by dry powder hepatitis B vaccine formulations administered by insufflator and aerosol generator. Mice were immunized twice on day 0 and day 14 with 2 μg of influenza subunit vaccine powder formulation by intramuscular (i.m.) or pulmonary route. Pulmonary powder formulations were administered by Penn-Insufflator [Pul Pow (PI)] or aerosol generator [Pul Pow (AG)]. Immune responses were determined on day 14 and day 28. Serum IgG titers on day 14 (A) and day 28 (B). (C) Serum IgG1 or IgG2a titers; (D) Lung IgG titers. Data are presented as average ± standard error of the mean ($n = 6$). Levels of significance are denoted as $^{**}P \leq 0.01$ and **** or $^{\#\#\#\#}P \leq 0.0001$.

powders induce equivalent immune responses upon deposition in trachea/central airways or deep lungs. However, a study by Minne et al.[7] reported deposition of influenza vaccine in the deep lungs to be superior to deposition in central airways. Deep lung deposition was hypothesized to be superior to upper or central airways due to improved residence time of the antigen as a consequence of reduced mucociliary clearance. However, the differences in immune responses reported in this study were only found to be significant between intranasal and deep lung targeted animals[7]. Moreover, only liquid influenza vaccine formulation was used; the physical state of the formulation and the delivery devices used for administration can also influence deposition of the vaccine formulation and thus the immune response. Therefore, the results of studies of Bhide et al.[9] and Minne et al.[7] cannot be directly translated to our current study.

Though deep lung targeting does not seem to be essential for influenza vaccine, the same cannot be concluded for hepatitis B vaccine. No immune responses were induced when hepatitis B powders were deposited in the trachea/central airways whereas the same powder led to considerable immune responses upon deposition in the deep lungs. Lombry et al.[24] studied the efficacy of DNA vaccines, in particular liquid hepatitis B surface antigen vaccine, by using two different administration routes. In this study, it was found that intranasal administration did not result in the generation of any immune response whereas intratracheal administration did. Since the aim of this study was only to investigate efficacy but not the optimal site of hepatitis B deposition in the respiratory tract, no conclusions were drawn about it. However, the observed differences found after administration *via* different routes could be ascribed to the volume of the vaccine solution used in the study of Lombry et al.[24] Based on the low volume used for intranasal administration, it is not expected to have resulted in deep lung deposition whereas the combination of high volume and air bolus in the intratracheal administration would have caused deep lung deposition[24]. In addition, Thomas et al.[25] and Minz et al.[26] administered liquid micro-particles or nanoemulsion containing hepatitis B antigen to the respiratory tract and it resulted in substantial humoral and cellular immune responses. Because of the high volumes of these formulations that were administered by intranasal and intratracheal route, we expect that both vaccine formulations were deposited deep in the lungs of rats. Minz et al.[26] concluded that deep lung deposition elicits stronger immune response; however, no head to head comparisons were made between immune responses elicited in upper airways and deep lung targeted animals. Thus, it would be unreasonable to draw the conclusion that deep lung deposition elicits a stronger immune response. In the study by Muttil et al.[2], hepatitis B powders were used. Upon dispersion from the insufflator, significant immune responses were induced in guinea pigs. This is in contrast to our study. However, the lungs of guinea pigs are much larger than those of the mice used in our study. Thus, the relative velocity at which powder particles entered the lungs must have been much lower. As a consequence, most likely no or very limited return flow of air occurred and therefore the powder may have been deposited for a part in the deep lung although they were administered *via* the insufflator. Though the aim of all these studies was not to investigate the influence of deposition on immune response, based on

experimental set-up and results, some conclusions regarding deposition could already be drawn by us.

For infectious diseases that are transmitted *via* the respiratory tract, such as influenza, measles and tuberculosis, both upper/central airways and deep lungs seem to lead to the induction of immune responses; however, in a number of studies these responses were found to be more robust when vaccine was delivered deep into the lungs[7,27–29]. Nevertheless, both upper/central airways and deep lung targeting has shown to induce protective efficacy against lethal viral challenge in pre-clinical animal models[9,27–29]. For these infectious diseases, influenza specific sialic acid, measles specific CD46 and tuberculosis specific pattern recognition receptors are present in the airways, which may play a role. In our study, despite of mucociliary clearance, comparable immune responses induced by powder particles deposited in trachea/central airways, can be explained by two reasons. Firstly, the spray-dried influenza formulation contains inulin in bulk amount, which will readily dissolve upon contact with the epithelial lining. Inulin, being an oligosaccharide, might substantially enhance the viscosity at the dissolution site thereby impeding mucociliary clearance. Secondly, sialic acid receptors are present in the upper airways of mice[30], these receptors might facilitate recognition of hemagglutinin, the major constituent of influenza subunit vaccine; thus, alleviating the need of deep lung targeting of influenza vaccine. Whether the uptake of influenza vaccine *via* these receptors leads to an immune response is, however, still not fully elucidated.

On the other hand, infectious diseases, such as hepatitis B, mainly spreads *via* infected blood or body fluids rather than respiratory tract. Hence, no hepatitis B specific receptors are present in the airways, neither in the upper nor in the deeper parts of the respiratory tract. Thus, the absence of hepatitis B specific receptors in these regions might have led to the removal of the vaccine by mucociliary clearance, despite of the high viscosity caused by the dissolution of inulin in the trachea/central airways; thus, no immune responses could be generated. However, the immune responses induced by deep lung targeting could be substantiated by two reasons. Firstly, Todoroff et al.[8] have provided an evidence of a prolonged residence time of the antigen following delivery to the deep lungs[8]; this provides ample time for interaction with immune cells to generate an immune response. Secondly, it is known that hepatitis B surface antigen forms virus like particles of about 22 nm and particles with a size <30 nm are known to passively diffuse from alveoli to draining lymph nodes to induce immune responses[31,32].

Interestingly, it seems that for airborne transmitted infections, diseases that are transmitted *via* the respiratory tract, deep lung deposition is not necessary to induce an immune response after pulmonary vaccination. On the other hand, it seems that for non-airborne transmitted infections, such as hepatitis B, deep lung deposition of the vaccine is essential to induce the desired immune response. From an evolutionary perspective it seems logical that the type of immune response after pulmonary administration varies between vaccines against airborne and non-airborne transmitted infectious diseases. Further research should investigate whether or not this hypothesis holds true for vaccine candidates against other pathogens that fall under these two categories. For example, vaccines against air-borne diseases such as pertussis, respiratory syncytial infection on one hand and against non-airborne diseases such as cholera, typhoid on the other could be compared. In addition, for respiratory tract transmitted diseases, the research should focus on the role of different receptors in the induction of immune responses upon deposition of vaccine formulations in trachea/central airways of pre-clinical animal models. In addition, it might also be interesting to investigate the biodistribution of fluorescently labeled vaccine at various time points. However, limitations like fluorescence attenuation and mucociliary clearance need to be taken into consideration for long term distribution experiments. These findings would have significant implications on the efficacy of pulmonary delivered vaccines at (pre-)clinical level.

5. Conclusions

In this study, we could achieve deposition of vaccine powder formulations in the trachea/central airways by insufflator and in the deep lungs *via* aerosol generator. Deposition site in the lungs seems to hold minor relevance for vaccines against infectious diseases that do spread *via* the respiratory tract whereas deep lung vaccine deposition is crucial for infectious diseases that do not spread *via* the respiratory tract. It might be due to the presence of influenza virus/vaccine specific sialic acid receptors and absence of hep-B-specific receptors in the respiratory tract of mice. Thus, optimal site of antigen deposition within the respiratory tract might depend on the type of antigen and the presence/absence of antigen specific receptors or binding sites in the respiratory tract. These pre-clinical results hold great potential for future clinical studies from a mechanistic point of view.

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